

Utilization of Cheese Whey and Cellulosic Biomass for Production of Ethanol by Selected Fungi Strain from Brazilian Savannas

Janaína Fischer^a, Verônica dos Santos Lopes^a, Célia M. A. Galvão^b, Juliana C. Teodoro^b, Ubirajara Coutinho Filho^a, Vicelma Luiz Cardoso^{*a}

^aFaculty of Chemical Engineering, Federal University of Uberlândia P.O. Box 593, Av. João Naves de Ávila, 2121-Bloco 1k, Campus Santa Mônica, Uberlândia-MG, Brazil

^bSugarcane Research Center (CTC), Farm Santo Antônio, without number, P.O. 13400-970, Piracicaba-SP, Brazil
 vicelma@ufu.br

The objective of this study was to produce ethanol using cheese whey, cellulosic biomass and crude enzymatic extract from wild-type fungi isolated from Brazilian Savanna (Cerrado). A total of 64 samples of material (flowers, fruit and soil) were collected and cellulase production by isolated fungal strains was evaluated using enzymatic activity index methodology.

The selected fungi was used to produce ethanol in a two step process: a) crude enzyme complex was produced by solid-state fermentation followed by the extraction of the enzyme complex from the fermented biomass using milk whey and hydrolyzed milk whey (0.1 % H₂SO₄, 0.5 atm, 30 min); b) enzymatic extract was used (Simultaneous Saccharification and Fermentation) to produce ethanol from *Saccharomyces cerevisiae* and steam-exploded sugarcane bagasse.

According to the results obtained, the use of cheese whey was effective in improving the ethanol yield. The use of cheese whey to extract enzyme complex increased the ethanol production in 20.4 % compared to a control test using water in place of cheese whey and the use of hydrolyzed cheese whey increased ethanol production in 55.1 % compared to water extraction. The best result for ethanol fermentation (17.9 g/L of ethanol) was obtained after 48 h of fermentation.

1. Introduction

Acknowledging the increasing demand for new fuel alternatives the substrate cost affects the overall cost and intensive scientific research to find substitute materials to reduce the substrate utilization are currently being studied in order to produce fuels (Binod et al., 2012; Zanette et al., 2008).

In this context, cellulose biomass emerges as an attractive raw material to produce ethanol, due to its abundant supply and low cost of production. The biomass used for fuel generation include forest by-products, sugar cane bagasse, wood residues, switchgrass, sweet sorghum, waste paper and other common agricultural wastes. Agricultural waste such as sugarcane bagasse is abundant in Brazil and has being widely investigated for its potential for producing of ethanol. Brazilian sugarcane harvest in 2011/12 was 560 million tons and for each ton of sugarcane processed, approximately 250 to 280 kg of bagasse is produced (MAPA, 2012; CTC, 2012).

A pretreatment step in ethanol production from lignocellulosic biomass required for converting cellulose to glucose and enzymatic hydrolysis has been proved to be more effective than the acid pretreatment (Castro and Pereira Jr., 2010). Although enzymatic hydrolysis offers the potential for higher yields the cellulases and related hydrolytic enzymes can represent about 18 % of total processing costs for biochemical conversion of cellulosic biomass feedstocks to sugars for biofuels (Zhuang et al., 2007).

Enzyme technology to produce second generation ethanol is one of the key areas to improve the hydrolysis of lignocellulosic materials. These processes have increased lately as research field undergoing to a great development in Brazil. Extensions of these research include microorganism isolation, identification and use for enhanced cellulolytic enzyme complex production and reduction of enzyme costs (Esterbauer et al., 1991; Haltrich et al., 1996; Lever et al., 2010).

In the screening for commercial enzymes niche habitats such as savannas are promising targets for research of microorganism for synthesizing cellulase enzymes. In this context the Brazilian Savanna (Cerrado), one of the largest biomes in the world, is important for its great potential for commercial enzymes. In this context, this paper analyses ethanol production of steam-exploded sugarcane bagasse and crude enzyme complex produced by fungi collected in the tropical savanna ecoregion of Brazil (Cerrado).

2. Experimental

2.1 Materials

A total of 64 samples of material (flowers, fruit and soil) from the Cerrado in the state of Minas Gerais (Uberlandia, Brazil) were collected and the isolate wild-type fungi strains. Species *Aspergillus niger* ATCC 16404, *Trichoderma reesei* CCT 2768 from the Culture Collection of the Fundação Tropical André Tosello (Brazil) were also used. The strains were kept on slants of Czapek agar stored at 5 ± 1 °C for 20 days and routinely subcultured onto same medium with the following composition (g/L): NaNO₃ 2.0; K₂HPO₄ 1.0; MgSO₄ 0.5; KCl 0.5; FeSO₄ 0.01; glucose 20.0; Agar 20.0.

All ethanol fermentations were performed using *Saccharomyces cerevisiae* Y904 obtained as lyophilized ethanol yeast from Mauri Brasil SA (Brazil). The yeast was stored in lyophilized form at 5 ± 1 °C for the entire study period.

The solid biomass used in the fermentations was rice byproduct milled and sieved through 1.8 mm mesh, whey powder reconstituted with distilled water to give a solid concentration of 60 g/L and sugarcane exploded sugarcane bagasse obtained from Sugarcane Research Center-CTC (São Paulo, Brazil). All biomass used was stored at 5 ± 1 °C for the entire study period.

2.2 Enzymatic activity

Enzymatic activity was evaluated using enzymatic activity index expressed by the relationship between the average diameter of the degradation halo and the average diameter of the colony growth when the cultures are incubated at 30 °C during 96 h in a solid medium with the following composition (g/L): MgSO₄ (0.5); KCl (0.5); NaNO₃ (3.0); FeSO₄.7H₂O (0.01); KH₂PO₄ (1.0); agar (15); carboxymethylcellulose (10.0) (Herculano et al., 2011).

2.3 Fermentations

Solid-state fermentations (SSF) to produce the enzyme complex were performed for 72 h at 30 °C in a 250 mL Erlenmeyer flask containing 10^7 to 10^8 cells/g of fungi, 40 g of solid medium with 24 g of rice bran and 16 g of exploded sugarcane bagasse (Rocha et al., 2013).

The ethanol fermentations were placed in 250 mL Erlenmeyer with 100 mL of fermentation broth containing exploded sugarcane bagasse, *Saccharomyces cerevisiae* Y904 and crude enzyme complex resulting from the extraction of enzymes from SSF with three different solutions (Table 1). The fermentations (pH 4.5, 30 g/L of inoculum) were incubated at 35 ± 1 °C and agitated at 150 rpm in a rotary shaker.

Table 1: Crude enzyme complex-extraction medium

Name	Description
MPET1	Tween 80 (0.1 g/L)
MPET2	Tween 80 (0.1 g/L) dissolved in milk whey
MPET3	Tween 80 (0.1 g/L) dissolved in hydrolyzed milk whey (0.1 % H ₂ SO ₄ , 0.5 atm, 30 min)

A comparative study of ethanol production by *Saccharomyces cerevisiae* Y904 using extraction medium MPET3 dissolved in enzymatic hydrolyzed milk whey (β -galactosidase, 3 g/L, pH 4.5, 35 °C, 2 h) and a comparative study using 16, 20 and 25 % (160, 200 and 250 g/L) of exploded sugarcane bagasse concentrations were also performed.

2.4 Analytical methodology

All fermentations were analyzed for ethanol and glucose production using high performance liquid chromatography (Shimadzu model LC-20A Prominence, Ca Supelcogel column) where the substances were detected by refractive index.

3. Results and discussions

3.1 Enzymatic activity

In the Figure 1 the results of the activity test of strains and wild-type fungi isolated from Cerrado (Brazilian Savanna) are showed. According to these results the strains 35, 38 and 42 and *Aspergillus niger* ATCC 16404 (fungi 65) showed to be a promising candidate for cellulase complex production. According to this results strain 42 was chosen to produce cellulase complex.

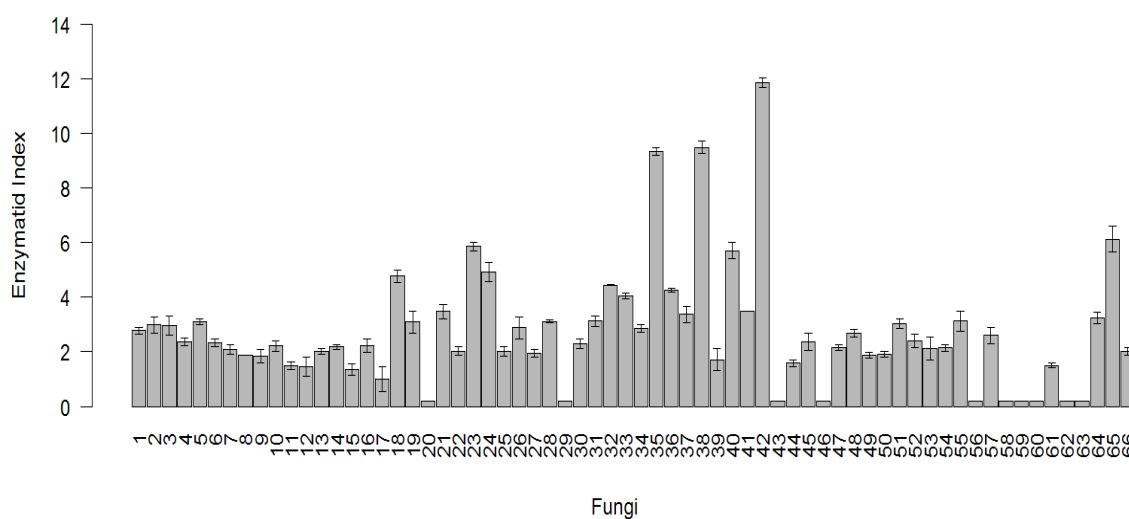


Figure 1: Enzymatic Activity Indices for 66 wild fungi strains grown on solid medium composed by agar with carboxymethylcellulose (CMC)

3.2 Fermentations

The Figure 2 represents glucose and ethanol production using enzyme complex produced in three different conditions of extraction (MPET1, MPET2 and MPTE3), steam exploded sugarcane bagasse (20 g/L) and *Saccharomyces cerevisiae* (30 g/L). According to the results no lag phase was observed in any of the media used. In addition minor changes in ethanol concentrations were observed during the stationary phase until 60 h. The ethanol production showed that the best operation condition for higher amount of ethanol production is achieved when hydrolyzed milk whey was used in enzyme extraction. Lever et al. (2010) reported that using crude enzyme extract produced by *Trichoderma reesei* CBS439.92 the final ethanol concentration reached 5.0 g/L after 96 h of SFS (simultaneous fermentation and saccharification) and when batch fermentation was substituted by fed-batch fermentation the final ethanol concentration reached 21 g/L after 168 h of fermentation. The ethanol concentration found in this work was higher than that observed in batch fermentation and lower than the observed in fed-batch fermentation.

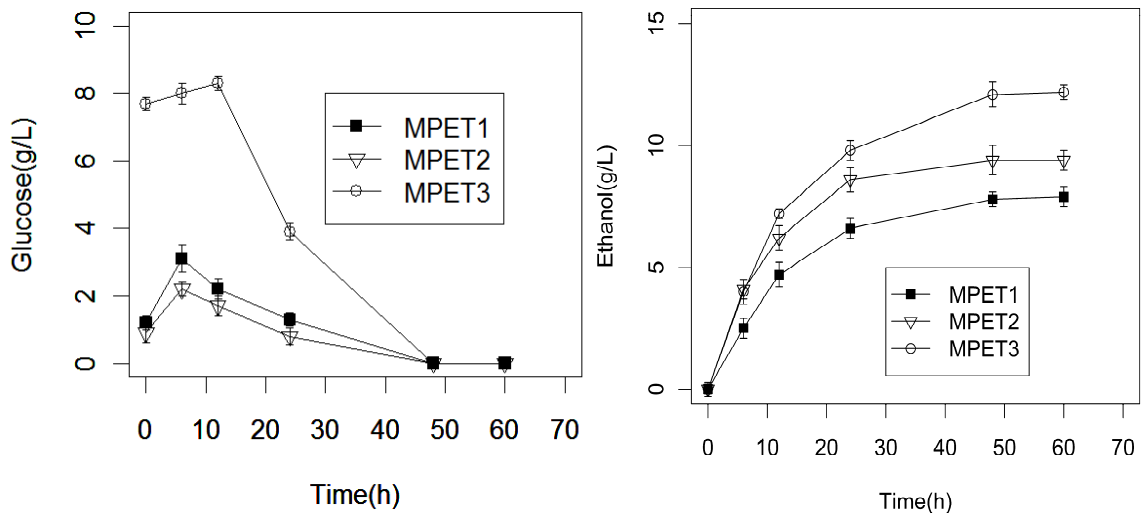


Figure 2: Simultaneous saccharification and fermentation

Ethanol concentrations are shown in Figure 3 for the formulations B16, B20 and B25 containing 16, 20 and 25 % (160, 200 and 250 g/L) of exploded sugarcane bagasse. A comparison between B16, B20 and B25 shows a significant increase in the ethanol production when the bagasse concentration is increased from 16 % to 20 % and significant decrease when the concentration increased from 20 % to 25 %. A comparison between these results with the results using cheese whey indicates that the ethanol concentration was influenced intensively by the initial bagasse concentration. For example, Dragone et al. (2011) using concentrated cheese whey powder reported 66.4 % of ethanol concentration found in B20.

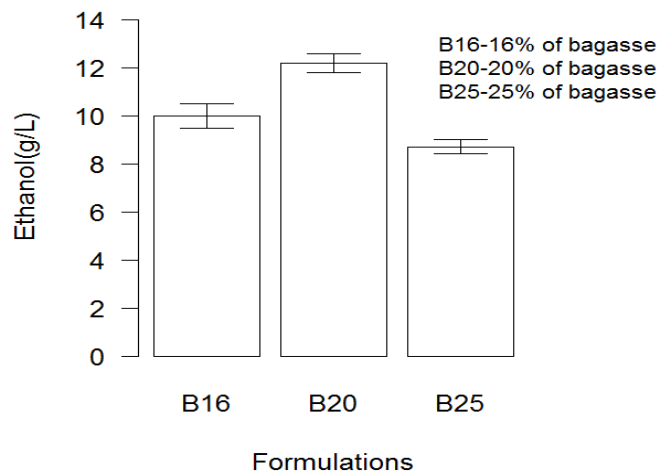


Figure 3: Ethanol concentrations for the formulations B16, B20 and B25 containing 16, 20 and 25 % (160, 200 and 250 g/L) of exploded sugarcane bagasse

The Figure 4 represents ethanol production using the formulations B20 and enzyme complex produced in MPTE3 condition of extraction with replace of acid hydrolysis by enzymatic hydrolyzed milk whey. The results from this figure indicate when the same extraction medium is used (MPET3) the enzymatic hydrolysis of lactose is more effective than acid hydrolysis (Figure 3). The result found was 90 % of that found by Oleskowicz-Popiel et al. (2012) for ethanol production from cheese whey co-digested with clover

grass containing 44 g/L of starch. This difference can be explained by the fact that starch fermentation is easier than cellulose fermentation.

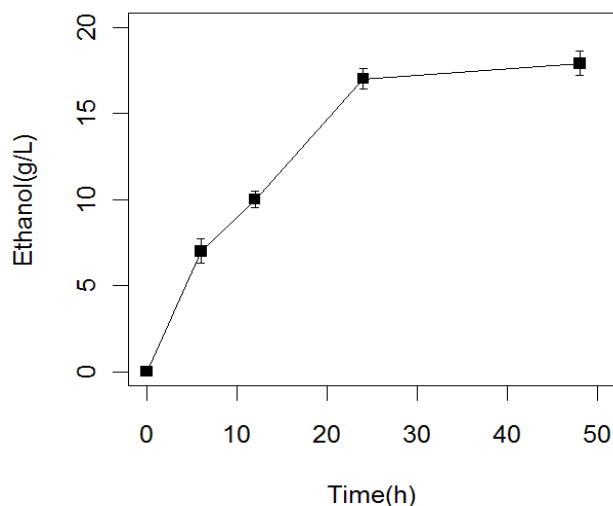


Figure 4: Ethanol production using extraction medium MPET3 dissolved in enzymatic hydrolyzed milk whey containing 20 % (200 g/L) of exploded sugarcane bagasse dissolved in MPET3

4. Conclusions

This study shows a potential use for selected fungi from Brazilian Savanna through the production of bioethanol. Other studies must be conducted to investigate the optimization of fermentation and hydrolysis by employing suitable nutrient and enzyme complex feeding strategies and co-digestion of other biomasses.

Acknowledgements

The authors wish to thank the CNPq (Edict 046/2009, Project 550940/2010-3), CAPES and FAPEMIG - Brazil – for the financial support and the CTC by the partnership in joint studies related to ethanol production.

References

- Binod P., Kuttiraja M., Archana M., Usha J.K., Sindhu R., Sukumaran R.K., Pandey A., 2012, High temperature pretreatment and hydrolysis of cotton stalk for producing sugars for bioethanol production, *Fuel* 92, 340-345.
- Castro A.M., Pereira Jr. N., 2010, Production, properties and application of cellulases in the hydrolysis of agro-industrial waste, *Química Nova* 33(1), 181-188 (in Portuguese).
- CTC (Centro de Tecnologia Canavieira, Brasil), 2012, <www.ctcanavieira.com.br> accessed 15.12.2012.
- Esterbauer H., Steiner W., Labudova I., Hermann A., Hayn M., 1991, Production of *Trichoderma* cellulose in laboratory and pilot Scale, *Bioresource Technology* 36, 51-65.
- Dragone G., Mussatto S.I., Silva J.B.A., Teixeira J.A., 2011, Optimal fermentation conditions for maximizing the ethanol production by *Kluyveromyces fragilis* from cheese whey powder, *Biomass and Bioenergy* 35, 1977-1982.
- Haltrich D., Nidetzky B., Kulbe K. D., 1996, Production of fungal xylanases, *Bioresource Technology* 58, 137-161.

- Herculano P. N., Lima D.M.M., Fernandes M. J. S., Neves R.P., Souza-Motta C.M., Porto A.L.F., 2011, Isolation of Cellulolytic Fungi from Waste of Castor (*Ricinus communis* L., *Current Microbiology* 62(5),1416-1422.
- Lever M., Ho G., Cord-Ruwisch R., 2010, Ethanol from lignocellulose using crude unprocessed cellulase from solid-state fermentation, *Bioresource Technology* 101, 7083-7087.
- MAPA (Ministério da Agricultura, Brasil), 2012, <www.agricultura.gov.br> accessed 10.12.2012.
- Oleskowicz-Popiel P., Kádár Z., Heiske S., Klein-Marcuschamer D., Simmons B.A., Blanch H.W., Schmidt J.E., 2012, Co-production of ethanol, biogas, protein fodder and natural fertilizer in organic farming – evaluation of a concept for a farm-scale biorefinery, *Bioresource Technology* 104, 440-446.
- Rocha N. R. F. A., Barros M.A., Fischer J., Coutinho Filho U., Cardoso V.L., 2013, Ethanol production from agroindustrial biomass using a crude enzyme complex produced by *Aspergillus niger*, *Renewable Energy* 57, 432-435.
- Zanette M., Torresendi M., Bolzonella D., Fatone F., Cecchi F., 2008, Continuous bioethanol and biogas production from food processing waste, *Chemical Engineering Transactions* 14, 245-250.
- Zhuang J., Marchant M.A., Nokes S.E., Strobel H.J., 2007, Economic analysis of cellulose production methods for bio-ethanol. *Applied Engineering in Agriculture* 23, 679-687.